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Rapid identification of bacterial isolates from wheat roots by high resolution whole cell MALDI-TOF MS analysis

Maria Isabel Stets[a], Artur Soares Pinto Jr.[b], Luciano Fernandes Huergo[a], Emanuel Maltempi de Souza[a], Vandeir Francisco Guimarães[b], Alexessander Couto Alves[c], Maria Berenice Reynaud Steffens[a], Rose Adele Monteiro[a], Fábio de Oliveira Pedrosa[a], Leonardo Magalhães Cruz[a,*]

[a] Department of Biochemistry and Molecular Biology, Federal University of Paraná (UFPR), CP 19046, 81.531-990 Curitiba, PR, Brazil
[b] Centro de Ciências Agrárias, Western Paraná State University (UNIOESTE), PR, Brazil
[c] Laboratory of Artificial Intelligence and Computer Science, University of Porto, Porto, Portugal

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ABSTRACT

Whole-cell mass spectrometry analysis is a powerful tool to rapidly identify microorganisms. Several studies reported the successful application of this technique to identify a variety of bacterial species with a discriminatory power at the strain level, mainly for bacteria of clinical importance. In this study we used matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) to assess the diversity of wheat-associated bacterial isolates. Wheat plants cultivated in non-sterile vermiculite, under greenhouse conditions were used for bacterial isolation. Total cellular extracts of 138 isolates were analyzed by MALDI-TOF MS and the mass spectra were used to cluster the isolates. Taxonomic identification and phylogenetic reconstruction based on 16S rRNA gene sequences showed the presence of *Pseudomonas*, *Pantoea*, *Acinetobacter*, *Enterobacter* and *Curtobacterium*. The 16S rRNA gene sequence analyses were congruent with the clusterization from mass spectra profile. Moreover, MALDI-TOF whole cell mass profiling allowed a finer discrimination of the isolates, suggesting that this technique has the potential of differentiating bacterial isolates at the strain level.

1. Introduction

Wheat is the second most important crop with a world production (2010/2011) of 651.6 million metric tons (USDA, 2011). Several works have identified bacteria associated with wheat plants, focusing on the so called plant growth-promoting bacteria (Beneduzi et al., 2008; Park et al., 2005; Sachdev et al., 2010), that potentially increase plant growth and crop production (Adesemoye et al., 2010; Baset Mia et al., 2010). Understanding the diversity of plant-bacterial associations is important for future biotechnological applications if these associations are to be manipulated to increase crop production, conserve biodiversity and sustain agro-ecosystems (Germida et al., 1998). However, the diversity of bacterial isolates from the plant surfaces or inner tissues can be overwhelming, and frequently many bacteria are very closely related. Finer profiling of bacteria associated with plants requires high throughput methods such as the next generation sequencing. However, the resolving power of next generation sequencing approach is rarely at the species level but never below. The drawback of such approaches is that the bacteria are not isolated, thus their biotechnological potential cannot be measured.

Whole-cell matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) or intact-cell MALDI-TOF (ICM) analysis (Dieckmann et al., 2005) is a powerful tool for rapid and highly resolving identification of microorganisms. This approach is a fast alternative for the identification of bacterial species and has been successfully applied to identify several microorganisms including viruses (Franco et al., 2010; Ganova-Raeva et al., 2010), fungi (Stevenson et al., 2010; Marklein et al., 2009; Schmidt and Kallow, 2005), insects (Campbell, 2005) and helminths (Perera et al., 2005). The technique is based on detection of molecular weight of most abundant cell molecules (presumably proteins and peptides) and using the mass spectra information as a fingerprint for a particular organism (Sauer and Kliem, 2010). Most of the recorded peaks in whole-cell mass spectra were assigned to ribosomal proteins. Although variability in the spectral profiles due to different factors have been reported (Šedo et al., 2011; Welker

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; CHCA, alpha-cyano-4-hydroxycinnamic acid; BBH, BLAST best hit; OTU, operational taxonomic unit; Da, Dalton.

* Corresponding author. Tel.: +55 0 21 41 3361 1657.
E-mail address: leonardo@ufpr.br (L.M. Cruz).

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and Moore, 2011), the method is reproducible for a given species (Welker and Moore, 2011). Minimal sample preparation and the speed of data acquisition combined with the high throughput and sample processing automation, make MALDI-TOF MS a valuable screening and identification method (De Bruyne et al., 2011).

Despite its potential, studies applying whole-cell MALDI-TOF MS to analyze microbial diversity to environmental samples are few. Dieckmann et al. studied bacterial isolates from marine sponges of the Sula Ridge by MALDI-TOF resolving 456 isolates into 11 taxonomic groups identified by 16S rRNA gene sequencing (Dieckmann et al., 2005). The authors showed that MALDI-TOF allows rapid grouping of the isolates, a critical step in comprehensive microbial studies of environmental samples or screening programs when large numbers of strains are isolated on different growth media (Dieckmann et al., 2005). The approach allowed rapid identification of isolates and selection of strains representing rare species for subsequent characterization. Furthermore, Ghyselinck et al. (2011) examined the suitability of MALDI-TOF MS and repetitive element sequence based polymerase chain reaction (rep-PCR) for the rapid grouping of bacterial isolates from the rhizospheres of potato plants. Cluster analysis and inspection of the profiles showed that for 204 isolates (82%) the taxonomic resolution of both techniques was comparable. Additionally, 16S rRNA gene sequence analysis was performed for all members of each cluster to gain insight into the identity and sequence similarity between members in each cluster. Munoz et al. (2011) used MALDI-TOF MS to classify bacterial isolates of solar saltern sediments into 25 phenotypic clusters at 52% similarity. The phylogenetic inference, made by 16S rRNA sequence analysis of the gene of selected strains, indicated that each phenotypic cluster comprised a genealogically homogeneous set of strains. Recently, MALDI-TOF MS was used to identify Rhizobiaceae with high accuracy (Ferreira et al., 2011), suggesting that this technique can be successfully used for studies of environmental diversity.

Furthermore, whole-cell MALDI-TOF MS is a powerful technique for biotechnological applications. Its capability to rapidly characterize microorganisms has potential applications in a number of areas such as medical diagnostics, biodefense, environmental monitoring, agricultural stewardship, food quality control, occupational safety, and culture typing (Demirev and Fenselau, 2008). For example, strain selection with potential use as biofertilizer depends on culture-dependent diversity studies, where a large number of bacterial isolates need to be screened by biochemical and molecular assays in order to proceed with taxonomical identification and characterization of its biotechnological potential. The technique may also be used to monitor population dynamics of plant growth-promoting bacteria in field experiments. In this study we compared MALDI-TOF MS and 16S rRNA gene sequencing to assess the diversity of bacteria associated with wheat plants.

2. Materials and methods

2.1. Wheat plants

Non-sterile wheat seeds cultivar BRS208 were grown in non-sterile vermiculite for 13 days under greenhouse conditions at 20 °C and 12 h photoperiod. Ten seeds per pot were sown and, after emergence, only four seedlings were maintained. Plants from 6 pots were pooled to make a composite sample for bacteria isolation.

2.2. Bacterial isolation

Wheat roots were washed in sterile water, weighed and ground in sterile saline solution (1:10, p/v) using a mortar and pestle until a homogeneous suspension was obtained. Serial dilutions of the root extract were plated onto Potato Dextrose Agar (Difco Laboratories Inc., USA) and incubated at 30 °C for 24 h. All colonies grown at dilution of 10^{-4} were subjected to mass spectrometry analysis. However, MALDI-TOF signals were not observed for all colonies directly analyzed. Those colonies were further purified by streaking them out to single colonies on a plate and subjected to a second round of spectrometry analysis using the same protocol.

2.3. Sample processing and MALDI-TOF mass spectrometry

We tested different factors that might influence the quality of spectra including the cell lysis method, MALDI matrix, culture age, culture media, and number of cells. The optimized protocol for extraction and preparation of bacterial samples for MALDI-TOF-MS analysis was as follows: isolated colonies from a pure culture plate (on Potato Dextrose Agar) containing about 10^8 cells (1–2 days after plating) were washed twice with 1 mL of water, cells were lysed with 40 μL of a formic acid (35%):acetonitrile (50%) mixture and the suspension was centrifuged at 25,000 × *g* for 15 min. One microliter of the supernatant was manually deposited onto a stainless steel MALDI-TOF target plate (Bruker-Daltonics, Bremen, Germany) and allowed to dry at room temperature. One microliter of α-cyano-4-hydroxycinnamic acid (CHCA) 10 mg/mL in acetonitrile 50% and trifluoroacetic acid 2.5% was added to the dried samples and allowed to dry under air.

2.4. MALDI-TOF mass spectrometry analysis

Mass spectra were obtained using an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Measurements were performed in linear positive ion mode, using a nitrogen laser (337 nm) at 50 Hz frequency. The acceleration voltage was 20 kV, with delay time acquisition and mass range of 3000–20,000 *m/z*. Typical resolution within this mass range was better than 600. External calibration was performed prior to each analysis using a mix of insulin (5734.51 *m/z*), ubquitin I (8565.76 *m/z*) and cytochrome c (12,360.97 *m/z*) (Protein Calibration Standard I – Bruker Daltonics). Automated spectra acquisition was performed using the Auto Execute tool of FlexControl 3.0 (Bruker-Daltonics) with fuzzy control of laser intensity. For each sample a total of 1000 laser shots were accumulated in 100 laser shots steps in 10 different regions of the same sample. The raw data were converted into a peak list using FlexAnalysis 3.0 software (Bruker Daltonics, Bremen, Germany), peak picking was performed using the method centroid of the peak, height 80%, peak with 0.1 *m/z* and signal/noise greater than 3, followed by one round of base line subtraction and smoothing.

MALDI-TOF MS data analysis of isolated bacteria was by using SPECLUST (Alm et al., 2006). Initially, the program calculates the peak match score, giving the probability those two peaks originated from the same peptide. The clustering procedures calculate the similarity score for each pair of peak lists, followed by calculating distances based on the similarity scores. Finally, a linkage procedure was applied to merge the clusters and construct the dendrogram. The SPECLUST parameters were set as follows: “liberal distance” for spectra grouping; “average” for distance calculation between groups; and a mass tolerance error of ±8 Da

2.5. Amplification, sequencing and analysis of 16S rRNA genes

Colony-PCR was performed using universal 16S rRNA gene primers 27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-ACGGCTACCTTGTTACGACTT-3′ (Lane, 1991). Cycling conditions were as follows: 2 min at 94 °C, 30 cycles of 45 s at 94 °C, 45 s at 60 °C, 1 min and 15 s at 72 °C, and a last extension step at 72 °C for 10 min PCR products were treated with 5.2 U and 0.77 U of

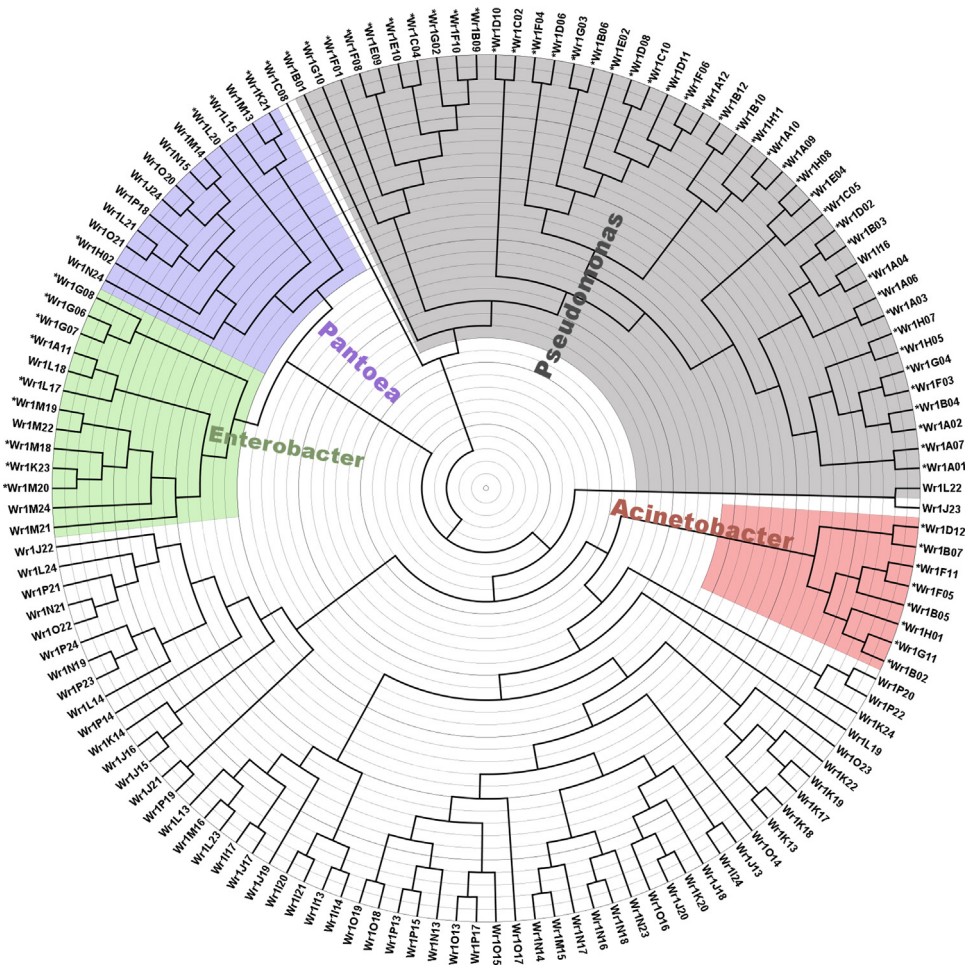

Fig. 1. Dendrogram for 138 bacteria isolates from wheat roots based on whole-cell MALDI-TOF MS spectra. Shaded groups cover isolates with taxonomic identification determined (marked with an asterisk). The dendrogram was constructed using the average linkage method from calculated distances using correlation similarity metric in SPECLUST software; uncertainty measurement was set as 5.0 Da; genera identification was achieved based on partial 16S rRNA gene sequences using RDP Classifier.

Exonuclease/shrimp alkaline phosphatase (Fermentas), respectively, and incubated at 37 °C for 1 h. The enzymes were heat inactivated at 80 °C for 30 min.

Amplified 16S rRNA gene fragments from cultured bacteria were partially sequenced using both 27F (Lane, 1991) and 805R (5′-GACTACCAGGGTATCTAAT-3′) (Soares-Ramos et al., 2003) primers and DYEnamic ET terminators (GE Healthcare) in an ABI 377 DNA sequencer (Applied Biosystems). A pre-analysis, including base calling, base quality checking, and trimming was carried out using Phred version 0.20425 (Ewing and Green, 1998), followed by sequence assembly using Phrap version 0.990319 (www.phrap.org). Visualization and manual edition of the consensus sequences were made using consed version 12.0 (Gordon et al., 1998). The consensus sequences of about 700 bp in length were used for taxonomic assignment and similarity search using RDP II Classifier tool (Wang et al., 2007) and MEGA-BLAST search (Zhang et al., 2000) against the NCBI "16S ribosomal RNA database (Bacteria and Archaea)". The obtained 16S rRNA gene sequences were then used to construct a phylogenetic tree with MEGA5 software (Tamura et al., 2011) and the Neighbor-Joining algorithm. The 16S rRNA gene sequences were deposited in the GenBank database (www.ncbi.nlm.nih.gov) under the accession numbers JF487991–JF488052 and JN258691–JN258702.

3. Results and discussion

Wheat plants of cultivar BRS208, cultivated under greenhouse conditions, were harvested on the 13th day after germination, the roots were macerated and subjected to the bacterial isolation procedure on Potato Dextrose Agar medium. A total of 1.2×10^6 bacterial colonies per gram of fresh roots were obtained, and 138 bacterial isolates were recovered after growth for 24 h and 30 °C.

All bacterial isolates from wheat roots were subjected to whole-cell MALDI-TOF MS analysis and the obtained spectra were clustered using the Speclust algorithm (Alm et al., 2006), resulting in the dendrogram shown in Fig. 1. Triplicate spectra from *Azospirillum brasilense* FP2 reference strain were used to calibrate the technical sample variation of MALDI-TOF MS and the mass tolerance error in SPECLUST as ±8 Da Representative spectra profiles of the bacterial isolates associated to wheat roots are shown in Fig. 2.

We also obtained the partial sequence of the 16S rRNA genes from 65 isolates distributed among the MALDI-TOF MS clusters and their taxonomic identity was accessed by RDP II Classifier tool (Wang et al., 2007). All isolates, except one, belong to the Gammaproteobacteria class of Proteobacteria phylum, distributed among five families: Enterobacteriaceae, with 2 genera and 13 identified isolates; Pseudomonadaceae, with 1 genus and 43 identified isolates; and Moraxellaceae, with 1 genus and 8 identified isolates.

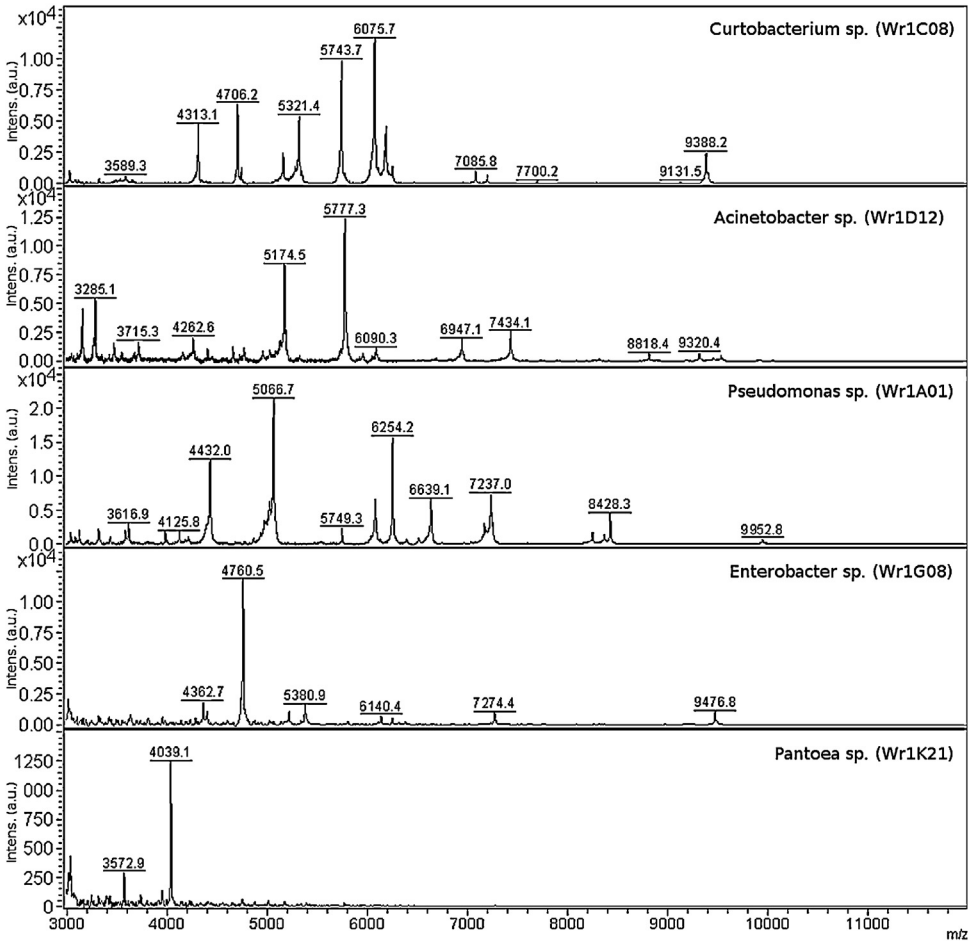

Fig. 2. Representative whole-cell MALDI-TOF MS spectra of bacteria associated to wheat plant roots. Genus assigned by 16S rRNA gene sequence followed by isolate identification in parenthesis are shown at the top of each spectra.

Pseudomonas was the predominant genus, but *Pantoea*, *Acinetobacter*, and *Enterobacter* were also found. One isolate (Wr1C08) was identified into the genus *Curtobacterium* from Actinobacteria phylum, a high GC, Gram-positive bacteria (Fig. 1). Although *Curtobacterium* constitute a distant related taxonomic group, it clusterized closer to *Pseudomonas*, not reflecting its phylogenetic relationship as showed by 16S rRNA-based tree (Fig. 3). It is well recognized that MALDI-TOF MS exhibit the potential to be a species-discriminating technique (Welker and Moore, 2011), however, the groups orientation in MALDI-TOF MS dendrogram does not necessarily reflect their evolutionary relatedness as revealed by genetic markers, such as 16S rRNA gene sequence (Dieckmann et al., 2005).

A MEGA-BLAST search against NCBI 16S ribosomal RNA database using the 5′ end of the 16S rRNA gene sequences showed little variation of the BLAST best hit (BBH) among isolate of the same cluster. The largest group of isolates (43 sequences) was identified as belonging to *Pseudomonas*, and the BBH was observed for *Pseudomonas libanensis* (GenBank accession number: NR_024901) with identities varying from 98% to 99%. The overall sequence identity of the aligned sequences in this group resulted in 99.1% identity, suggesting that this group of isolates is closely related, possibly strains of the same species. Similar results were observed for the other groups: the *Acinetobacter* cluster (8 sequences) showed a BBH for *Acinetobacter* [*calcoaceticus*] (GenBank: NR_042387) with 99% identity and overall identity of aligned sequences of 98.8%; *Enterobacter* (9 sequences) showed a BBH for *Enterobacter cowanii* (GenBank: NR_025566) with 98% to 99% identity and overall identity of aligned sequences of 98.8%; and *Pantoea* (4 sequences) showed a BBH for *Pantoea agglomerans* (GenBank: NR_041978) with 95% to 99% identity and overall identity of aligned sequences of 96.9%. The isolate WrC08 showed sequence similarity to *Curtobacterium flaccumfaciens* (GenBank: NR_025467) at 99% identity. These results suggest that the isolates of the same genus are very closely related, most of them probably belonging to the same species because of the high sequence similarity. Thus MALDI-TOF MS has higher resolving power than 16S rRNA sequences, possibly discriminating strains within specie level, since the isolates were separated using the mass profiles.

To demonstrate the reproducibility and taxonomic resolving power of mass spectrometry, three *Azospirillum* species commonly found associated to grass rhizosphere were analyzed by MALDI-TOF MS, namely: *A. brasilense*, *Azospirillum amazonense* and *Azospirillum lipoferum*. Six strains (Sp7, Sp245, FP2, HM210, SF0 and SF5) of *A. brasilense*, two strains (Y2 and Y6) of *A. amazonense* and only strain DSM 1691 of *A. lipoferum* were included in the analysis. Strains FP2 and HM210 are derivatives from *A. brasilense* Sp7, while SF5 and SF0 are derivatives from *A. brasilense* Sp245. The bacteria were grown in DYGS medium and analyzed in biological triplicates following the same procedures used for wheat isolates described in Material and Methods. The spectra were used for hierarchical clustering and the generated dendrogram is shown in Fig. 4. The three *Azospirillum* species were grouped into separated clusters and the four derivative strains of *A. brasilense* Sp7 and Sp245 also clustered according to their parent strains. It is noteworthy that the replicates always clustered together, but different strains formed distinct branches, distinguishing parent and derivative strains of *A. brasilense* (Fig. 4).

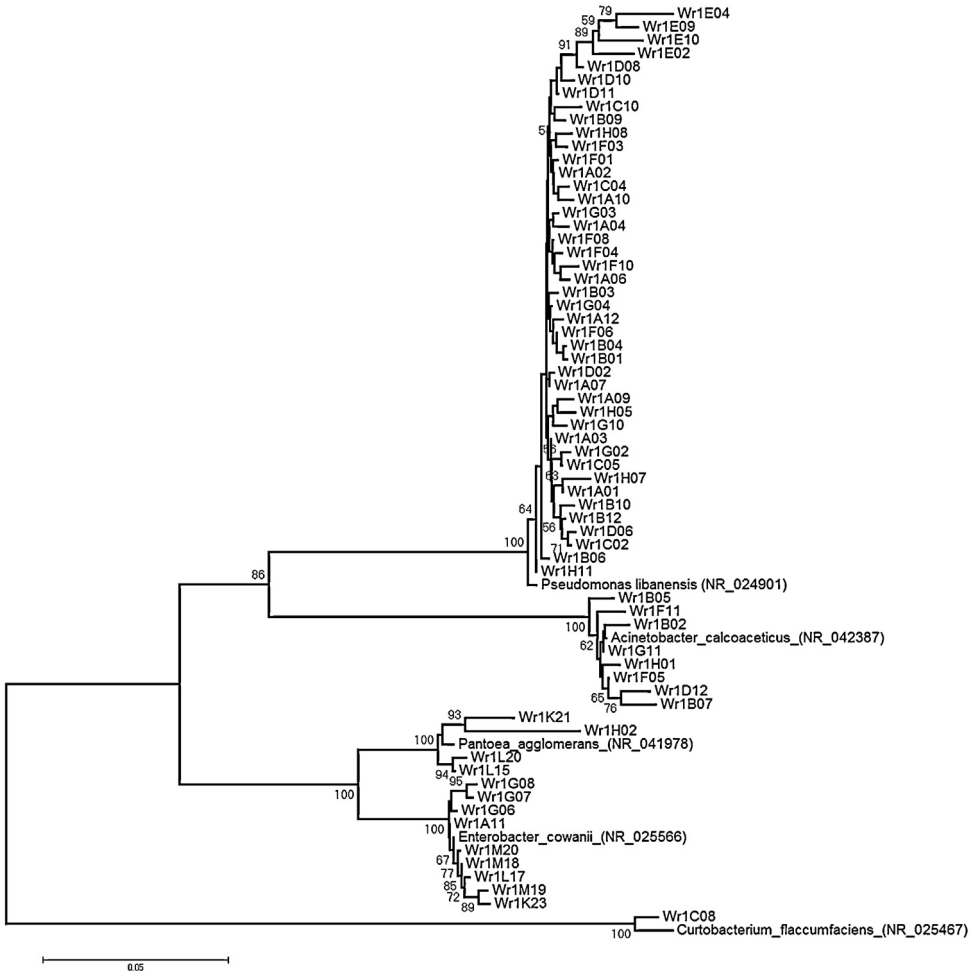

Fig. 3. Phylogenetic tree for 65 bacterial isolates based on partial 16S rRNA gene sequences. The tree was generated using the Neighbor-Joining method (Saitou and Nei, 1987) from Jukes–Cantor calculated distances (Jukes and Cantor, 1969); percentage of 10,000 bootstrap replicates are shown at each node; GenBank sequences of type strains of closely related species are included as references (accession numbers follow species name).

Our results agree qualitatively with previous studies by Sachdev et al. (2010) that observed *Pseudomonas* amongst the most dominant genera and also found *Acinetobacter* sp., *Stenotrophomonas* sp. and *Enterobacter* sp. when studying microbial diversity associated with wheat rhizospheres during flowering-stage of the plants. Egamberdiyeva (2007) in their study of salt-tolerant bacteria from the rhizosphere of Uzbek wheat also found the prevalence of *Acinetobacter* sp., *Pseudomonas* sp., *Enterobacter* sp. and *Pantoea*

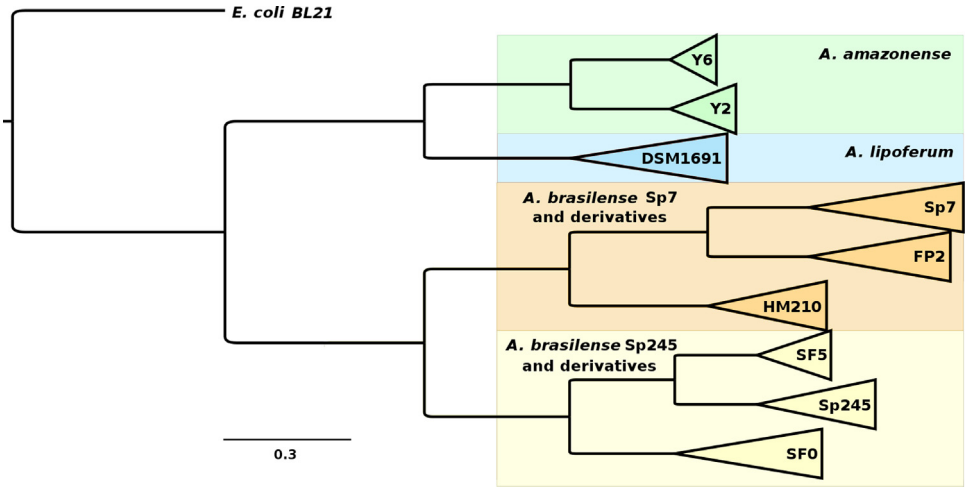

Fig. 4. Whole-cell MALDI-TOF MS spectra-based dendrogram for *Azospirillum* species and reference strains. The taxonomic discriminatory power at strain level is demonstrated for the technique; *A. brasilense* strains: Sp7, Sp245 (wild types), FP2, HM210 (Sp7 derivatives), SF5 and SF0 (Sp245 derivatives); *A. amazonense* strains: Y2 and Y6 (wild types); *A. lipoferum* strain DSM 1691; the dendrogram was constructed using the average linkage method from calculated distances using correlation similarity metric in SPECLUST software; uncertainty measurement was set as 5.0 Da; triangles account for triplicate variation.

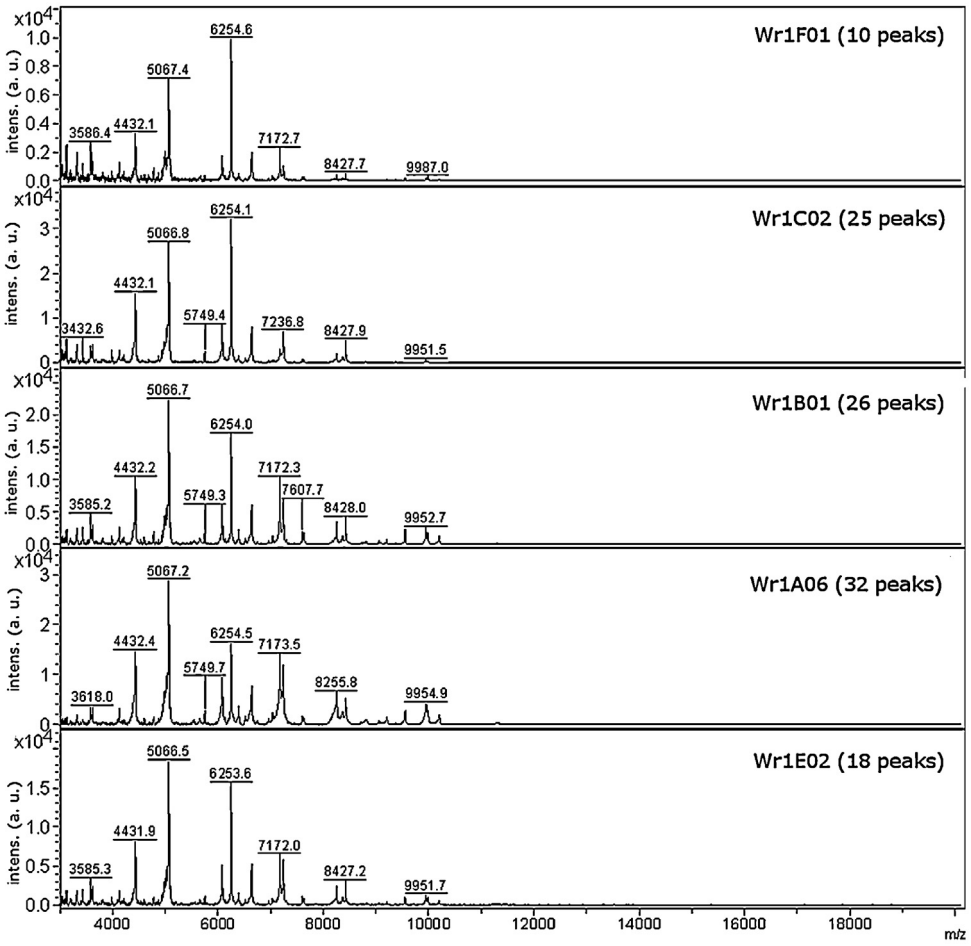

Fig. 5. Comparison of whole-cell MALDI-TOF MS spectra of representative *Pseudomonas* isolates. The identification of isolates are shown above each spectrum, followed by number of peaks detected in parenthesis.

sp. The *Pseudomonas* genus was also found as one of the predominant groups in endophytic bacteria isolated from sugarcane stems and coastal sand dune plants (Magnani et al., 2010; Shin et al., 2007).

A phylogenetic tree constructed using the 65 16S rRNA gene partial sequences and related sequences retrieved from the GenBank database (www.ncbi.nlm.nih.gov) show the sequences clustered according to the previous taxonomic assignment (Fig. 3). However, a low resolution, as shown by the low bootstrap values (42% of the tree nodes show bootstrap values above 50) was observed within genera clades, possibly due to the high identity shared by the sequences.

The dendrogram derived from whole-cell MALDI-TOF MS profiling agreed well with the phylogenetic tree based on 16S rRNA gene sequences. However, comparison of the dendrograms obtained with whole-cell MALDI-TOF MS analysis and 16S rRNA gene sequence phylogeny it is clear that the former has a higher resolution within the genus level than the latter, separating the isolates sharing high 16S rRNA sequence identity in different clusters. This higher resolution by MALDI-TOF MS profiling had been previously reported for bacterial isolates from marine sponges where *Pseudoalteromonas* sp. isolates differing in only 1 bp out of 400 bp or by 3–4 bp out of 1500 bp of their 16S rRNA gene sequences could be readily discriminated by their MALDI-TOF MS spectra (Dieckmann et al., 2005). Furthermore, analysis of spectra profiles obtained by MALDI-TOF MS of a seafood isolate allowed its identification as *Pseudomonas fragi* due to the presence of characteristic peak masses and the absence of peak masses specific for other *Pseudomonas* species, whereas 16S rRNA gene sequence analyses did not separate these species because of to the high sequence similarity (Böhme et al., 2010).

In our work, a total of 43 sequenced isolates were assigned as *Pseudomonas*. Comparison of the MS spectra obtained for these isolates revealed the presence of a total of 50 peaks (data not shown). Of these, only 8 peaks were present in 100% of the isolates and 14 peaks were present in more than 90% of the isolates. On the other hand, 11 peaks were characteristic of only one isolate and 18 peaks were present in fewer than 10% of the isolates. Fig. 5 shows the peak profile of the most peak-abundant isolate (Wr1A06 with 32 peaks) compared to least peak-abundant isolate (Wr1F01 with 10 peaks). However, sufficient information was obtained in peak profiles to group closely related organisms in different clusters.

MALDI-TOF MS has been extensively applied for identification of clinical isolates by using comprehensive spectra databases of reference pathogenic strains. Proprietaries software are then used to search the database from a peak list of clinical isolates to assign them taxonomically. Applications of this technology in environmental studies have also being developed. Ferreira et al. (2011) showed that MALDI-TOF profiling can be used to differentiate species of the family Rhizobiaceae. The authors used the program Biotyper (Bruker Daltonics) to build a database of profiles of 56 species of fast growing rhizobia species and were able to identify isolates from nodules. Ziegler et al. (2010) obtained MALDI-TOF profiles directly from nodules of cowpea, soybean or siratro, and were able to identify inoculated strains, indicating that this technique has a potential to identify bacteria without previous isolation.

A potentially attractive application of MALDI-TOF profiling is quality control of commercial inoculants of nitrogen-fixing and plant growth-promoting bacteria which can take advantage of fast sample preparation, low cost and high discriminatory power.

Potential complications of MALDI-TOF mass spectrometry profiling bacterial growth conditions, sample processing and data acquisition that can significantly influence the mass spectra obtained (Liu et al., 2007). Different environmental conditions, such as culture media, can produce different results due to changes in bacterial metabolism (Rajakaruna et al., 2009; Keys et al., 2004; Valentine et al., 2005; Salaün et al., 2010). However, Conway et al. (2001) observed that *Escherichia coli* grown in two different broths yielded spectra 80% similar which is enough to correctly identify *E. coli*. To verify the effect of the culture media on the reproducibility of the mass spectra, we cultivated the same *A. brasilense* strain in four different media: Potato Dextrose Agar (Difco), DYGS (Rodrigues Neto et al., 1986), LA (Sambrook et al., 1989) and NFbHP containing either malate or lactate as carbon source (Machado et al., 1991). Bacterial colonies were analyzed by MALDI-TOF MS as before. Spectra were 77.3% similar among replicates of the same medium, however the similarity dropped to 37.9% when all five different media were compared. We also observed that some media are more informative than others, producing spectra with higher number of picks. DYGS presented about 40% more picks than LA and about 18% more picks than NFbHP and Potato Dextrose Agar. The results suggest that, contrary to *E. coli*, for correct profiling of *Azospirillum* strains it is necessary to use the same medium.

Identification of environmental bacteria poses a huge challenge since the diversity of bacterial species in natural environments can be very high. In addition, the proportion of unknown/non-cultured bacteria is very high in many niches such as soil and plant rhizospheres. These factors make the taxonomic identification of environmental bacteria by whole-cell MALDI-TOF MS using the standard technique based on searching the spectra database of reference strains difficult at the present time. However, the hierarchical clustering of spectra from isolates constitutes an alternative for dereplication and identification of environmental isolates (Dieckmann et al., 2005; Ghyselinck et al., 2011). The high resolution of MALDI-TOF MS analysis allows rapid clustering of large libraries of isolates to select representative strains for 16S rRNA gene sequencing. This is particularly important when screening large collections of isolates for biotechnological use since the desired activity may vary enormously at intra-specific level, thus discrimination among different strains would serve to direct screening approaches. For example, screening of plant growth-promoting rhizobacteria involves isolation and evaluation of large collection of isolates, requiring grouping of strains of genetically similar bacteria for further analyzes.

In conclusion whole-cell MALDI-TOF MS analysis can be used as a rapid and efficient screening method for grouping bacterial isolates from environmental samples in operational taxonomic units (out), independently of databases and previous known taxonomic positions. This technique in combination with sequencing of 16S rRNA of selected isolates was applied to culture-dependent analysis of the bacterial diversity associated with wheat roots, revealing a prevalence of the Gammaproteobacteria, with *Pseudomonas* and *Pantoea* as the main groups. In addition the results showed that MALDI-TOF profiling can provide a finer resolution compared to partial 16S rRNA gene sequencing, discriminating isolates at species/strain level.

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